A 106.24:S-12
ISSN 0193-3728

Alternaria macrospora as a Potential Biocontrol Agent for Spurred Anoda

Production of Spores for Field Studies

U.S. Department of Agriculture
Science and Education Administration
Advances in Agricultural Technology • AAT-S-12/April 1980

The research reported in this publication was done in cooperation with the Mississippi Agricultural and Forestry Experiment Station.

The author thanks J. A. Riley and L. B. Turfitt, biological technicians, Southern Weed Science Laboratory, for technical assistance.

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This publication is available from the Southern Weed Science Laboratory, P.O. Box 225, Stoneville, Miss. 38776.

Science and Education Administration, Advances in Agricultural Technology, Southern Series, No. 12, April 1980.

Published by Agricultural Research (Southern Region), Science and Education Administration, U.S. Department of Agriculture, P.O. Box 53326, New Orleans, La. 70153.

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Alternaria macrospora as a Potential Biocontrol Agent for Spurred Anoda

Production of Spores for Field Studies

By H. Lynn Walker [1]

ABSTRACT

The method described produces sufficient *Alternaria macrospora* Zimm. spores to permit field evaluation of this fungus as a biocontrol agent for spurred anoda [*Anoda cristata* (L.) Schlecht.]. Mycelia grown in liquid culture are blended, poured into pans, and incubated under a specific light-dark regime. Dried spores are harvested with cyclone spore collectors and stored under refrigeration at 4° C or frozen at -10° C. Spore preparations produced by this method contain $2\text{-}4\times10^8$ spores per gram and can be stored for as long as 12 months and still maintain over 90% viability. The method has been shown to be applicable for the production of spores for several other fungi. Index terms: *Alternaria macrospora* Zimm., biological control, fungi, spore production, spurred anoda, weed control.

INTRODUCTION

Spurred anoda [*Anoda cristata* (L.) Schlecht.], a member of the Malvaceae, infests an estimated 810,000 hectares (2 million acres) in the Southern United States (Chandler and Oliver 1979). The weed is increasing in importance in southern crops, particularly cotton and soybeans, and it is both difficult and expensive to control with currently registered herbicides (Chandler and Oliver 1979).

In 1974 the fungi *Alternaria macrospora* Zimm. and *Puccinia heterospora* Berk. & Curt. were involved in an epiphytotic on spurred anoda in the Yazoo-Mississippi Delta region (Ohr et al. 1975). *A. macrospora* isolated from diseased spurred anoda plants killed spurred anoda seedlings under greenhouse conditions, and the fungus was cited as a potential biocontrol agent (Ohr et al. 1977). In preliminary host-range studies the spurred anoda isolate of *A. macrospora* did not significantly damage representative cotton varieties that are grown in the southwestern and southern United States (Walker and Sciumbato 1979a). In contrast, cotton pathogenic isolates did not infect spurred anoda, but incited moderate to severe damage on the cotton varieties tested (Walker and Sciumbato 1979b).

Field testing of the spurred anoda isolate of *A. macrospora* as a potential biocontrol agent has been limited by the amount of inoculum that could be produced. This paper describes a production method that yields sufficient spores to permit field-scale inoculation and evaluation. A similar method was previously described for the production of *Pyricularia oryzae* Cav. spores (Latterell 1975). Briefly, Latterell's method involves the production of mycelia in liquid shake cultures. The mycelia are separated from the liquid, placed on wire screen, and incubated under

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continuous light for 2 to 3 days in a cabinet at 26° C and 97% to 98% relative humidity. The spores are harvested with an organic solvent, dried, processed through a screen, packaged, and frozen in liquid nitrogen at -10° C or refrigerated at 4° C. Stored in this manner, the spores remained highly viable for as long as 16 years. The method described here is similar in that mycelia are first produced in liquid shake cultures, then induced to sporulate under controlled environmental conditions. However, the sporulation requirements for this isolate of *A. macrospora* and the method of spore harvest are quite different.

SPORE-PRODUCTION METHOD

A. macrospora used in this study was isolated from diseased spurred anoda plants collected near Stoneville, Miss. The fungus was isolated on PDA and sporulated profusely in petri dishes of V-8 juice agar (Miller 1955) incubated at 24° C with 12 h diurnal light. The light source was two 15-W cool-white fluorescent lamps suspended 45 cm above the cultures. V-8 juice agar consists of 3 g $CaCO_3$, 15 g agar, 200 ml V-8 juice, and enough distilled water to make 1 liter of final volume.

Spores from a 7- to 10-day-old culture were suspended in 2 to 3 ml sterile distilled water and used to inoculate 500 ml sterile modified Richard's medium (Daniel et al. 1973) with 1% sucrose contained in 1,000-ml Erlenmeyer flasks plugged with cotton. This medium was previously sterilized by steam autoclaving at 1,055 g/cm² for 45 min. The inoculated flasks were incubated on a rotary shaker at ambient laboratory conditions at 110 revolutions per minute (r/min). Modified Richard's medium consists of 10 g commercial sucrose, 10 g KNO_3, 5 g KH_2PO_4, 2.5 g $MgSO_4 \cdot 7H_2O$, 0.02 g $FeCl_3$, 150 ml V-8 juice, and enough distilled water to make 1 liter of final volume. The pH is adjusted to 6.0 with 50% (w/v) NaOH.

One of the 500-ml cultures, 24 to 48 h old, was used as inoculum for 7 liters of modified Richard's medium with 1% sucrose contained in 10-liter Kimax carboys plugged with cotton. These carboys of growth medium were previously steam-autoclaved at 1,055 g/cm² for 60 min. After inoculation, the carboys of growth medium were incubated on rotary shakers at 110 r/min at ambient laboratory conditions (fig. 1).

After 24 h, numerous spherical colonies of fungus were apparent throughout the medium and ranged in size up to 5 mm in diameter. After 72 h, the colonies ranged in size to 10 mm in diameter. Each carboy was harvested by collecting the contents onto plastic-coated nylon window screen. Aseptic conditions were not maintained during the harvest of the mycelia and the remainder of the procedure.

FIGURE 1.—Carboys containing liquid shake cultures of *Alternaria macrospora*. These cultures are 72 h old.

FIGURE 2.—Aluminum-foil-lined plastic pan into which mycelial homogenate of *Alternaria macrospora* is being poured.

To each wet volume of 500 ml of mycelia were added 200 ml of distilled water and 3 ml of an antibiotic solution containing streptomycin sulfate (3.7 mg/ml) and chloroamphenicol (2.5 mg/ml). This mixture was blended for 30 s at high speed in a Waring Blendor.

Each 250 ml of the mycelial homogenate was mixed with an additional 100 ml of distilled water, then poured into an aluminum-foil-lined plastic pan measuring 41 by 27 by 5.5 cm (fig. 2). Pans of mycelial homogenate were placed on a Plant Mobile (Wards Natural Science Establishment, Inc.). Each Plant Mobile held 3 rows of 4 pans (fig. 3), and each carboy culture produced 7 to 10 pans of mycelial homogenate. After all pans were in place, the Plant Mobile was covered with the black plastic enclosure provided by the manufacturer (fig. 4). Two 12-cm-square sections were removed from the top of the enclosure to allow ventilation. Lighting was supplied by two 40-W cool-white fluorescent lamps suspended 45 cm above each Plant Mobile shelf. The quantum flux density was 62 μEin/m^2/s (400-700 nm) as measured with a Lambda PAR meter. The mycelial homogenate, incubated at ambient laboratory temperatures, received an initial 7 h of light, followed by 13 h of darkness, followed by 4 h of light. The temperature within the plastic-draped Plant Mobile ranged from 25° C during the dark periods to 32° C during the light periods. The relative humidity ranged from 80% to 90%.

Alternating light was found to be critical for spore production. Spores were not produced under conditions of continuous light or continuous darkness. Forty-watt cool-white fluorescent lamps induced spore production, whereas 40-W Gro-Lux fluorescent lamps and 40-W incandescent lamps did not induce adequate spore production. Black light has been reported to induce abundant sporulation in another isolate of *A. macrospora* (Bhama and Balasubramaniam 1977). In our work, 40-W fluorescent black lights and 60-W incandescent black lights induced sporulation but were no better than cool-white fluorescent lamps. In some tests, pans of mycelial homogenate received a 15-min exposure to 500-W Photoflood incandescent lamps before placement in an unlit incubator at 25° C with 98%-100% relative humidity. Sporulation was observed. Without the light treatment, there was no sporulation.

After 24 h the surfaces of the mycelial layers in the pans were black to dark olive green and were covered with conidia and conidiophores. The pans were removed from the Plant Mobile and placed in

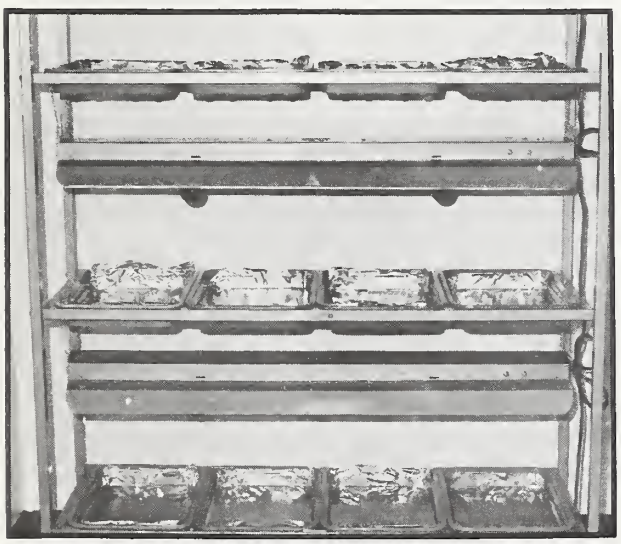

FIGURE 3.—Plant Mobile with pans of blended *Alternaria macrospora* mycelia.

FIGURE 4.—Plant Mobile with plastic cover.

unlighted incubators at 25° C for 24 h to allow the spores to mature. The relative humidity during this period was maintained at 90% to 100%.

After the 24-h maturation period, the pans were removed from the incubator and allowed to air-dry. The dried mycelial layers were smooth and paperlike in texture.

The spores were harvested with two cyclone spore collectors (Trevet and Cherry 1950) that were connected in series to a small vacuum pump with adjustable suction (fig. 5).

The spores were stored in closed containers and refrigerated at 4° C or frozen at -10° C.

RESULTS AND DISCUSSION

Spore Yield

Mycelial yield was determined from carboy cultures 72 h old. The mycelia were collected onto plastic-coated nylon window screen, rinsed with distilled water, placed into tared weighing trays, and dried to constant weight at 75° C. The dry weight of mycelia per carboy was found to be 26 to 28 g.

Each of our pans yielded up to 1.3 g of spores, with most pans producing 0.75 to 1.0 g (figs. 6 and 7). These preparations contained $2\text{-}4\times10^8$ spores per gram, as determined with a hemacytometer. Each 7 liters of growth medium produced about 7.5 g of spores, or about 0.3 g of spores per gram of mycelia.

We have consistently produced 80 to 100 g of spores per week by processing 11 to 13 carboy cultures. At an experimental inoculum level of 500 g of spores per hectare (200 g/acre), which has been shown to be a realistic spore concentration under laboratory and field conditions, sufficient inoculum has been produced in the laboratory to enable field evaluation of *A. macrospora* for the biological control of spurred anoda.

This method is far more efficient regarding time, labor, and materials than spore production methods based on petri-dish cultures. For comparison, *A. macrospora* cultures grown in petri dishes of V-8 juice agar for 7 to 14 days yield $4\text{-}6\times10^6$ spores per dish, or approximately 0.02 g of spores. Therefore, the spore yield from each carboy after 4 or 5 days is equal to the yield of 375 to 400 petri-dish cultures 7 to 14 days old.

Spore Viability

Samples were periodically removed from storage and checked for viability. The spores were placed in distilled water containing 0.1% Tween 80[2] in a deep well slide and incubated without a cover slip at 25° C for 6 h. The percentage of spores with germ tubes was calculated from random fields of view with a compound microscope at 200 × magnification. Multiple germ tubes from a single spore were often observed, but a spore was counted as viable only once whether one or several germ tubes were observed. Spores stored under the conditions described maintained over 90% viability for as long as 12 months.

[2]Oxysorbic (20 POE) (polyoxyethylene sorbitan monooleate).

Figure 5.—Cyclone spore collectors used to harvest *Alternaria macrospora* spores.

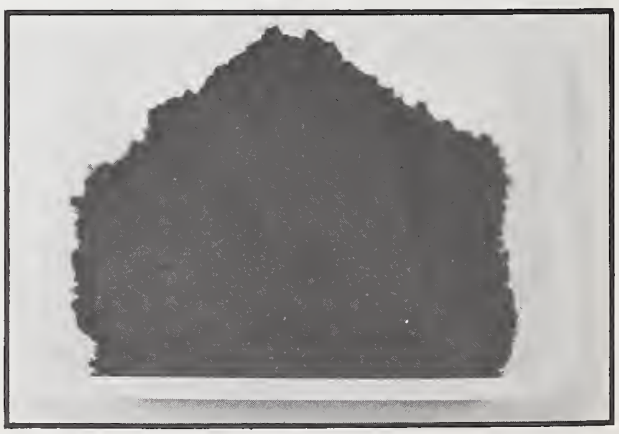

Figure 6.—Final spore product after harvest. Approximately 150 g is shown.

Spore Infectivity

Spores produced as described were suspended in distilled water containing 0.1% Tween 80 and sprayed onto 12 greenhouse-grown spurred anoda seedlings in the 2- to 5-leaf stage. These plants, plus 12 control plants sprayed with distilled water and 0.1% Tween 80 only, were placed in a dew chamber (Percival I-35D) with an air temperature of 25° C. After 10 to 16 h in the dew chamber, the plants were moved to a greenhouse bench. The greenhouse temperature was maintained at 28° to 35° C. The quantum flux density was 1,650 μEin/m^2/s (400-700 nm) at noonday. The relative humidity remained between 60% and 85%.

The spores were highly infective. Inocula containing 5,000 to 10,000 spores per milliliter of inoculum, as determined with a hemacytometer, produced typical target-spot lesions on the leaves and stems within 3 to 5 days. Inoculum concentrations of 300,000 to 500,000 spores per milliliter consistently killed spurred anoda seedlings within 3 to 5 days.

CONCLUSIONS

This method can possibly be adapted to produce spores of many other fungi where aseptic conditions are not required. It has been successfully used in our laboratory with several other fungi: *Colletotrichum graminicola* (Ces.) G. W. Wils., *Colletotrichum malvarum* (A. Braun and Casp.) Southworth, *Drechslera sorghicola* (Lefebvre and Sherwin) Richardson and Fraser, *Helminthosporium* spp., and *Curvularia* spp. It is likely that spore yields per volume of growth medium can be improved significantly through better culture conditions.

Figure 7.—Photomicrograph of spore preparation. × 700.

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☆U.S. GOVERNMENT PRINTING OFFICE: 1980-671-123/38A

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